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Assessment of peripheral nerve pathology in laminin-alpha2-deficient mice.

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Authors	Jeffrey Boone Miller, Mary Lou Beermann, and Sachiko Homma Neuromuscular Biology & Disease Group, Boston University School of Medicine, USA
Working group members	Madeleine Durbeej (Faculty of Medicine, Lund University, Lund, Sweden)
SOP responsible	Jeffrey Boone Miller
Official reviewer	Madeleine Durbeej

TABLE OF CONTENTS

1. OBJECTIVE.....	3
2. SCOPE AND APPLICABILITY	3
3. CAUTIONS	4
4. MATERIALS	4
5. METHODS	6
5.1 Sciatic Nerve Immunohistochemistry.	6
5.2 Ventral root histology	11
6. EVALUATION AND INTERPRETATION OF RESULTS.....	13
7. REFERENCES	14
8. ACKNOWLEDGEMENTS	15

1. OBJECTIVE

We describe histological methods for quantitative assessment of peripheral nerve pathology, including aberrant Schwann cell differentiation, in laminin-alpha2-deficient mice. In addition to skeletal muscle and brain pathologies, human MDC1A patients show signs of peripheral neuropathy such as a slowed nerve conduction velocity (Shorer et al., 1995; Muntoni and Voit, 2004; Reed, 2009; Collins and Bönnemann, 2010, Rocha and Hoffman, 2010). Similarly, laminin-alpha2-deficient mice, including *Lama2* knockouts, also show pathological changes in skeletal muscles, brain, and peripheral nerves (Nakagawa et al., 2001; Guo et al., 2003). In this section of the SOP, we describe methods to analyze peripheral neuropathy, whereas other authors in this series will describe methods to analyze pathology in skeletal muscle and white matter of the brain.

In particular, we describe methods to quantify peripheral nerve pathology and Schwann cell differentiation status, using lumbar ventral roots and sciatic nerves as examples. The methods include toluidine blue staining and cellular analyses of ventral root sections, as well as immunohistochemical and morphometric analyses of frozen sections of sciatic nerves to identify areas within the nerves that have abnormal extracellular matrix, myelin, or axonal neurofilaments. Furthermore, to analyze the status of Schwann cell differentiation in laminin-alpha2-deficient mice, which is known to be abnormal (Bray and Aguayo, 1975; Stirling, 1975; Bray et al., 1977; Perkins et al., 1980; Jaros and Jenkinson, 1983; Patton et al., 2008), we describe immunohistochemical assays with differentiation stage-specific markers – including glial fibrillary acidic protein (GFAP), Oct6, and Krox20 – to quantify immature/non-myelinating, pre-myelinating, and mature/myelinating Schwann cells respectively (Jessen et al., 1990; Zorick et al., 1996; Arroyo et al., 1998; Svaren and Meijer, 2008).

2. SCOPE AND APPLICABILITY

The purpose of this SOP is to describe methods to quantitatively assess peripheral nerve pathology, including Schwann cell differentiation status, in laminin-alpha2-deficient mice. The assays are designed to be suitable both for quantifying the state of pathology in untreated mice and for determining if experimental therapeutic strategies can limit or reverse peripheral nerve pathology.

This SOP is written for laboratories with the necessary equipment, personnel, and expertise to carry out immunohistological and morphometric assays of embedded, fixed, or frozen sections of mouse nerve tissues. We will describe the methods and antibodies required for quantitative cellular and immunohistochemical analyses, but we will not focus on methods of dissection or sectioning which are general techniques that are easily

adaptable to peripheral nerve tissue. The methods described here are limited to cellular analyses of dissected tissue at the resolution of light and fluorescence microscopy. We do not describe methods for electron microscopic analyses or for physiological analyses of living nerves (e.g., conduction velocity), though both types of analyses are of significant value in assessing peripheral nerve pathology.

3. CAUTIONS

Numbers of mice required. As might be expected, there is considerable mouse-to-mouse variability in peripheral neuropathology that we have found is on the order of $\pm 20\%$. The extent of pathology also depends on the age of the mouse that is examined, and gender should also be considered. Investigators should carry out a power analysis to determine the numbers of mice that will need to be examined to determine if differences between two groups (e.g., treated and untreated) are statistically significant. Previous studies suggest that experimental and control groups comprised of 8-10 mice of a single age will be needed [e.g, Patton et al., 2008; Homma et al., 2011], as is also the case for muscle and brain pathology.

Selection and handling of tissues. Though the methods presented here were developed through analyses of sciatic nerve and lumbar ventral root tissues, the methods should be applicable to any peripheral nerve tissue. Because anatomy of nerve structures can vary from mouse to mouse or at different locations within the structure, it is important to standardize the dissection(s) so that comparisons among animals or treatment groups will be valid. For example, if the sciatic nerve is to be analyzed, then the same region of the nerve (e.g., a 1 cm length from mid-thigh to knee) should be dissected from each animal. If ventral roots are to be analyzed, then roots from a standard spinal level (e.g., lumbar 2) should be used. Dissected tissues should be handled so that sections can be made at the same orientation (e.g., transverse) for all samples. Throughout the dissections, it is important to handle the tissues gently so that overall structure is maintained.

Safety. Follow all manufacturer's recommendations for safe use of reagents and instruments, as well as all local safety, biosafety, and environmental regulations.

4. MATERIALS

As noted above, this protocol assumes that the investigator has access to a laboratory that is equipped for mouse dissection, tissue sectioning, and light microscopy (including fluorescence microscopy). Once the tissue sections are prepared in the proper manner, the investigator will also require access to collections of primary antibodies (Table 1) and

MDC1A_M.1.2.001

fluorescent secondary antibodies (Table 2), as well as additional chemicals, reagents, and supplies (Table 3).

Table 1: Primary Antibodies.

Target Protein	Catalog Number Source	Antibody Type Working dilution
Glial Fibrillary Acidic Protein (GFAP)	MAB360 Millipore, Billerica MA	Mouse mAb 1:1000
Ki67	VP-K451 Vector Laboratories, Burlingame, CA	Rabbit pAb 1:1000
Krox20	PRB-236P Covance, Princeton NJ	Rabbit pAb 1:100
Laminins (multiple subunits)	L-9393 Sigma-Aldrich, St. Louis MO	Rabbit pAb 1:50
Myelin Basic Protein	MAB386 Millipore, Billerica MA	Rat mAb 1:500
Neurofilament H (phosphorylated)	SMI-31R Covance, Princeton NJ	Mouse mAb 1:1000
Oct6	sc-11661 Santa Cruz Biotech, Santa Cruz CA	Goat pAb 1:50

Table 2: Fluorescent Secondary Antibodies.

Antibody Type	Catalog Number; Source; Working dilution
Donkey anti-goat Alexa 488	A11001; Invitrogen, Carlsbad CA; 1:500
Goat anti-mouse Alexa 488	A11001; Invitrogen, Carlsbad CA; 1:500
Goat anti-rabbit Alexa 488	A11008; Invitrogen, Carlsbad CA; 1:500
Goat anti-rabbit Alexa 594	A11012; Invitrogen, Carlsbad CA; 1:500
Goat anti-rat Alexa594	A21209; Invitrogen, Carlsbad CA; 1:500
Goat anti-rat Cy3	112-165-006; Jackson ImmunoResearch West Grove PA; 1:500

Table 3. Additional chemicals, reagents, and supplies.

Name	Source
Cryomolds, Tissue-Tek	Sakura Finetek USA, Torrance CA
CytoSeal 60 mounting medium	Electron Microscopy Science, Hatfield PA
DAPI (4',6-diamidino-2-phenylindole)	Invitrogen, Carlsbad CA
Embed 812 kit, #14120	Electron Microscopy Science, Hatfield PA
Glutaraldehyde (25% solution, EM grade)	Electron Microscopy Science, Hatfield PA
Molds, flat embedding	Electron Microscopy Science, Hatfield PA
Normal goat serum	Vector Laboratories, Burlingame CA
Normal rabbit serum	Vector Laboratories, Burlingame CA
Normal rat serum	Vector Laboratories, Burlingame CA
O.C.T. Tissue-Tek tissue freezing medium	Sakura Finetek USA, Torrance CA
Osmium Tetroxide	Electron Microscopy Science, Hatfield PA
Paraformaldehyde, 16% solution, EM grade	Electron Microscopy Science, Hatfield PA
Propylene Oxide, EM Grade	Electron Microscopy Science, Hatfield PA
Superfrost Plus microscope slides	Electron Microscopy Science, Hatfield PA
Toluidine Blue O	Electron Microscopy Science, Hatfield PA
Vectashield H-100 mounting medium	Vector Laboratories, Burlingame CA

5. METHODS

5.1 Sciatic Nerve Immunohistochemistry

Preparation of tissue sections

Immediately upon dissection, place ~0.5 – 1 cm long segments of sciatic nerves in standard phosphate-buffered saline (PBS). After dissections are complete, embed the nerve segments in O.C.T. Tissue-Tek using cryomolds to orient the segments in an appropriate orientation for transverse sections, and immediately snap-freeze on dry ice. Prepare frozen sections (7 – 10 μm) with a cryostat and place sections onto one or more Superfrost Plus microscope slides in a pattern that will allow identification of adjoining sections if needed. Air dry the slides at room temperature for 60 minutes or overnight, and either use immediately or store the slides at -80°C until needed. Remove the slides from the freezer

and allow to equilibrate at room temperature for 30 min. Place the slides in washing racks, and wash gently with PBS three times for 5 minutes each to remove O.C.T.

Fixation and blocking

Single immunofluorescence. The fixation conditions required for different single antibodies are listed in Table 4. The fixatives are either ice cold 100% methanol (MeOH) for ten minutes at 4°C or 3.7% paraformaldehyde (PFA) in PBS for ten minutes at room temperature as noted in Table 4. After fixation, wash the slides three times for 5 minutes each in PBS at room temperature. After washing, incubate with the appropriate blocking buffer as listed in Table 4. Blocking buffer for single staining is either normal goat serum buffer (3% BSA, 1% normal goat serum, 0.3% Triton X-100 in PBS) or normal rabbit serum buffer (3% normal rabbit serum, 0.3% Triton X-100 in PBS) as noted in Table 4. Incubate slides in blocking solution for 1 hour at room temperature in a humidified chamber.

Table 4. Conditions for performing single immunofluorescence assays with the primary antibodies described in Table 1 (see text for double immunofluorescence conditions).

Target	Fixation*	Blocking Buffer **	1° antibody incubation
Glial Fibrillary Acidic Protein (GFAP)	MeOH	Normal goat	Overnight at 4°C
Ki67	3.7% PFA	Normal goat	1-2 hours at room temperature or overnight at 4°C
Krox20	MeOH	Normal goat	2 hours at room temperature or overnight at 4°C
Laminins (multiple subunits)	3.7% PFA or MeOH	Normal goat	30 min at room temperature
Myelin Basic Protein	MeOH	Normal goat	60 min at room temperature
Neurofilament H (phosphorylated)	3.7% PFA or MeOH	Normal goat	45 min at room temperature
Oct6	3.7% PFA	Normal rabbit	Overnight at 4°C

*To detect the target protein listed here with the corresponding primary antibody from Table 1, fix sciatic nerve sections as indicated with either 3.7% paraformaldehyde (PFA) for 10 min at room temperature or ice cold Methanol (MeOH; 100%) for 10 min at 4°C.

**Normal goat serum blocking buffer = 3% BSA, 1% normal goat serum, 0.3% Triton X-100 in PBS. Normal rabbit serum blocking buffer = 3% normal rabbit serum, 0.3% Triton X-100 in PBS.

Double immunofluorescence. Double staining using pairs of antibodies is carried out using fixation and blocking conditions that depend on the pair of primary antibodies to be used as follows:

(i) **Double stain for neurofilaments and laminins.** Fix with 100% MeOH. After fixation, wash the slides three times for 5 minutes each in PBS at room temperature. Block with normal goat serum blocking buffer (3% normal goat serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature in a humidified chamber. Either anti-neurofilament antibody or anti-laminin antibody can be used as first or second primary antibody.

(ii) **Double stain for laminins and myelin basic protein.** Fix with 100% MeOH. After fixation, wash the slides three times for 5 minutes each in PBS at room temperature. Block with normal goat serum blocking buffer (3% normal goat serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature in a humidified chamber. The first primary antibody is anti-laminin antibody (incubate for 0.5-1 hour at room temperature) and the second primary antibody is anti-myelin basic protein (incubate for 1 hour at room temperature).

(iii) **Double stain for Oct6 and Krox20.** Fix first with 3.7% PFA and then with 100% MeOH. After fixation, wash the slides three times for 5 minutes each in PBS at room temperature. Block with normal rat serum blocking buffer (3% normal rat serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature in a humidified chamber. The first primary antibody is anti-Oct6 antibody (overnight incubation at 4°C) and the second primary antibody is anti-Krox20 antibody (incubate for 2 hours at room temperature).

(iv) **Double stain for Oct6 and Ki67.** Fix first with 3.7% PFA and then permeabilize with 1% Triton X-100 in PBS for 10 min at room temperature. After fixation, wash the slides three times for 5 minutes each in PBS at room temperature. Block with normal rat serum blocking buffer 1 hour at room temperature in a humidified chamber. The first primary antibody is anti-Oct6 antibody (overnight incubation at 4°C) and the second primary antibody is anti-Ki67 antibody (incubate for 2 hours at room temperature).

Primary and secondary antibody incubations, nuclei staining, mounting, and microscopy

Single antibody immunostaining. After blocking, incubate the slides with the primary antibody diluted in the same blocking buffer (for incubation times and temperature refer to

Table 4). The working dilutions are listed in Table 1. After incubation, wash three times in PBS as described above. Incubate the slides with the secondary antibody for 1 hour at room temperature using a dilution of 1:500. At the end of the secondary antibody incubation, wash the slides three times with PBS as above. Incubate with 300 nM DAPI in PBS for 10 min at room temperature to stain nuclei, then wash three times in PBS (alternatively, it is possible to add DAPI with the secondary antibody). Add a small drop of Vectashield H-1000 anti-fade fluorescent mounting medium to each slide and apply glass cover slips as usual. Store slides in a dark and humidified chamber at 4°C.

Double antibody immunostaining. For the first antibody and its appropriate secondary antibody, follow same procedure as for single staining. Wash the sections 3 times for 5 minutes each with PBS and incubate with 2nd primary antibody at the appropriate dilution in blocking buffer as described in Table 4. After antibody incubation, wash three times in PBS, incubate the sections with the appropriate secondary antibody for 1 hour at room temperature. Add DAPI with the secondary antibody to stain nuclei. After three washes in PBS, add the anti-fade fluorescent mounting medium and coverslip.

Imaging. Examine the sections with a standard or confocal fluorescent microscope equipped with the proper filters for the fluorescent reporters used. Capture images of the sections with a digital imaging device and store the images for later analyses. Obtain both low power (4X, 10X) images that contain the entire section, as well as higher power images (20X, 40X) that allow analyses of individual cells.

Image analyses

1. Cross-sectional area of the sciatic nerve. From low power (e.g., 4X) images of sciatic nerve cross-sections (or collages of high power sections), use an appropriate method to determine the total cross-sectional areas of the nerve sections. Examples of imaging programs that can be used to measure cross-sectional area include SPOT Advanced v4.6 (Diagnostic Instruments, Sterling Heights, MI) and NIH Image (<http://rsbweb.nih.gov/ni-image/>). In our laboratory, we have found that the mean cross-sectional area of sciatic nerves from *Lama2^{dyW/dyW}* mice is approximately 70 – 75% that of wild-type littermates at four weeks after birth (Homma et al., 2011). It is important to be sure that the sections are transverse and not at an oblique angle. If the section is at an oblique angle (e.g., axon profiles appear generally oval with long axes uniformly pointed in one direction, rather than generally round), then it is possible to correct for the oblique angle by calculating the minimum distance of parallel tangents at opposing particle borders (i.e., minimal Feret's diameter) as described previously (Briguet et al., 2004).

2. Total number and density of nuclei. From DAPI stained sections, count the total number of nuclei per cross-section, which is typically on the order of 300 – 400 (Perkins et al., 1980). From the total number of nuclei and the cross-sectional area, calculate the

nuclear density per mm². The total number of nuclei per cross-section is typically approximately the same in both wild-type and laminin-alpha2-deficient sciatic nerves (Perkins et al., 1980, Homma et al., 2011). However, the nuclear density can be significantly higher in laminin-alpha2-deficient nerves due to the lower cross-sectional area.

3. Percentage of the nerve cross section area that has an abnormal neurofilament staining pattern or lacks staining for laminins and/or myelin basic protein. Use an image analysis program to identify, mark, and measure areas within each sciatic nerve section in which, depending on the antibodies used, (i) neurofilament organization is abnormal, (ii) staining with the anti-laminins antibody is absent, or (iii) staining for myelin basic protein is absent. Wild-type sciatic nerves will show a staining pattern in which each axon, identified by neurofilament staining, is outlined by laminins or myelin basic protein staining, whereas *Lama2*^{dyW/dyW} sciatic nerves will have regions of varying size that show disorganized neurofilaments that are not outlined by laminins or myelin basic protein as shown in Fig. 1. In our laboratory, typically 10 – 20% of the cross-sectional area will show a pathological pattern of antibody staining (Homma et al., 2011). Additional investigations are required, e.g., with laminin subunit-specific or extracellular matrix antibodies, to determine if particular laminin subunits remain in the pathological areas or if additional ECM components are affected.

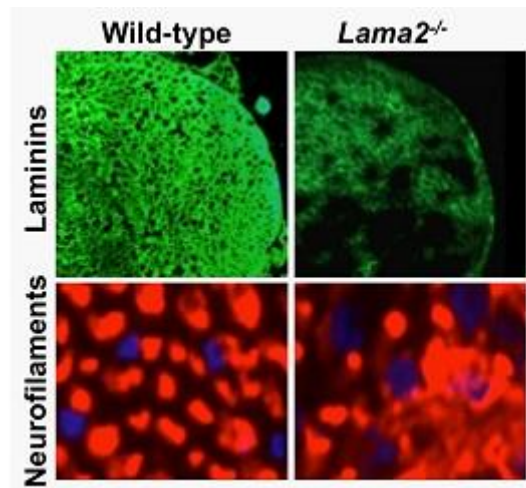


Fig. 1. Sections of 4 week-old wild-type and *Lama2*^{-/-} sciatic nerves were stained with antibodies specific for multiple laminins or neurofilaments as indicated. Low power views (upper row) show that the *Lama2*^{-/-} sciatic nerves have areas in which laminins are absent, whereas all wild-type axons are outline by laminins. High power views (lower row) shows areas of abnormal neurofilament organization in *Lama2*^{-/-}, but not wild-type, nerves.

4. Percentage of cells that express markers specific for different stages of Schwann cell differentiation. For sections stained for GFAP, Oct6, and Krox20, count the number of

MDC1A_M.1.2.001

cells that are and are not stained by each antibody and calculate the percentage of cells that express each protein. Oct6 and Krox20 staining is typically cytoplasmic, whereas GFAP staining is cytoplasmic. GFAP is a marker for immature/non-myelinating Schwann cells, Oct6 is a marker for pre-myelinating and pro-myelinating (i.e., in contact with axons) Schwann cells, and Krox20 is a marker for mature/myelinating Schwann cells. Sciatic nerves from wild-type and *Lama2^{dyW/dyW}* mice have different distributions of cell labeling. Oct6 and Krox20 are typically located in nuclei. Wild-type nerves have a low percentage of Oct6-positive nuclei and a high percentage of Krox20-positive nuclei, which is consistent with successful Schwann cell maturation and myelination. In contrast, *Lama2^{dyW/dyW}* nerves have a significantly higher percentage of Oct6-positive nuclei and fewer Krox20-positive nuclei, which is consistent with the decreased numbers of mature Schwann cells and areas of amyelination seen in laminin- α 2-deficient peripheral nerves (Bray and Aguayo 1975; Jaros and Bentley, 1978; Patton et al., 2008; Homma et al., 2011). Analysis of sections doubly stained for Oct6 and Krox20 can supplement the single staining experiments by identifying the percentages of nuclei that are positive only for Oct6, only for Krox20, for both, or for neither.

5. Percentage of cells that express the proliferation marker Ki67. As a marker for proliferating cells, determine the percentage of cells that express Ki67. Previous studies have shown that older (> 4 weeks old) wild-type sciatic nerves have very low levels of Schwann cell proliferation, whereas Schwann cell proliferation remains active in laminin- α 2-deficient mice of the same age (Perkins et al., 1980; Homma et al., 2011). Double staining for Ki67 and Oct6 will identify the percentage of Oct6-positive cells that remain proliferative.

5.2 Ventral root histology

Dissection and fixation

Upon dissection of the ventral roots from the chosen spinal level, e.g. lumbar 2 (L2), place roots in standard phosphate-buffered saline (PBS). After dissections are complete, fix the segments in 0.1M sodium cacodylate buffer at pH 7.4 containing 2% glutaraldehyde, 2% paraformaldehyde, and 3.5% sucrose for 2 h at 4 °C. After three washes in 0.1M sodium cacodylate buffer, postfix the tissue with 1% osmium tetroxide on ice for 2 h and wash three times with 0.1M sodium cacodylate buffer.

Dehydration

Working in an appropriate fume hood, dehydrate the roots in an ethanol (EtOH) and propylene oxide series, i.e., 50%, 70%, 80%, 90%, 95% EtOH for 10 min each; 100% EtOH for

20 min three times at room temperature; and 100% propylene oxide for 15 min at room temperature.

Infiltration

During the dehydration series, prepare the embedding mixture following the manufacturer's instructions with particular attention to recommended precautions for safe use. Mix 20 ml of EMBED812, 16 ml of DDSA, 8 ml of NMA and 0.66-0.88 ml of DMP30 (or the same proportion of these four components depending on the volume needed). Mix very well. After the embedding mixture is ready, drain the propylene oxide from the root samples, replace the solvent with a 2:1 solution of propylene oxide:embedding medium, and allow to stand for at least 1 hour at room temperature. Change the mixture to 1:1 then 1:2 solution of propylene oxide:embedding medium and leave for 1 hour for each. Remove the mixture, replace it with 100% embedding medium and leave for 2 hours at room temperature. Repeat twice.

Embedding

Transfer ventral roots to dry embedding mold and fill with embedding medium. Polymerize the medium in an oven at 60°C for 24 hours.

Sectioning and staining

Cut semi-thin sections (0.5-1.0 μ m) with an ultra-microtome, and use a steel loop to collect and transfer sections to a drop of distilled water on a slide. This will flatten sections to avoid wrinkles. Dry sections on a slide by placing the slide on a slide warmer or lamp. After the sections are completely dried, cover with a few drops of Toluidine blue solution (0.5% Toluidine Blue and 2% sodium Borate in distilled water, filter before use) for 2-5 min. During this staining, the heat source can remain on. Rinse off excess stain gently with distilled water. Coverslip with regular mounting medium after sections are completely dried.

Imaging

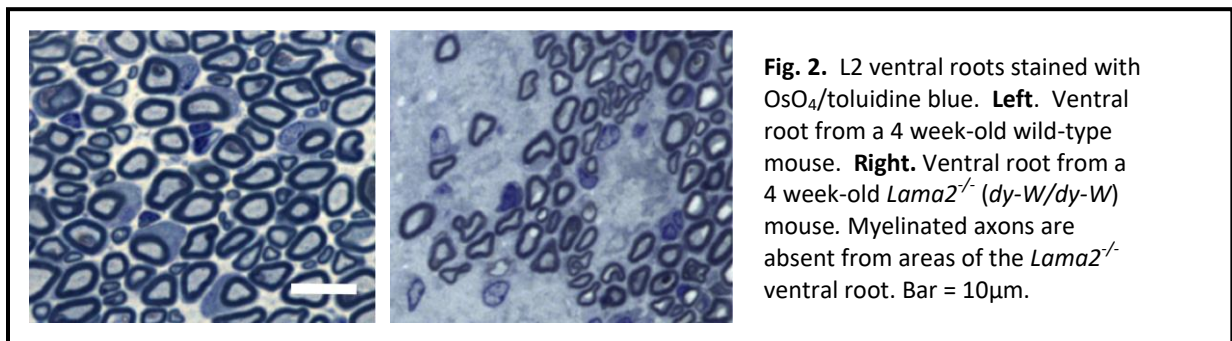
Capture images of the sections with a digital imaging device and store the images for later analyses. For later analyses, obtain both low power (4X, 10X) images that contain the entire section, as well as higher power images (20X, 40X) that allow analyses of individual cells, axons, and myelin sheaths.

Image analyses

Determine cross-sectional areas of ventral root sections as described above and count the number of myelinated axons within each root section. Transverse ventral root sections have an elongated oval shape (Biscoe et al., 1982). Myelinated axons within the roots are identifiable by the heavily stained myelin sheath that surrounds each axon (Fig. 2). For the L2 ventral root, for example, wild-type roots contain ~600 – 1000 well myelinated axons that

MDC1A_M.1.2.001

are evenly distributed throughout the root, whereas *Lama2*^{-/-} L2 ventral roots have significantly fewer myelinated axons (typically <200) and also show regions that lack myelinated axons (Fig. 2) (Biscoe et al., 1982; Homma et al., 2011). Investigators may also want to measure additional parameters such as myelin thickness and axonal diameters as described in Nakagawa et al., 2001, but those assays are beyond the scope of this protocol.



Summary

These assays described here are designed to quantify the extent of pathology within sciatic nerves and ventral roots of laminin-alpha2-deficient mice. Because wild-type and laminin-alpha2-deficient sciatic nerves and ventral roots show significant differences in the assayed properties, these assays can be used to evaluate the effectiveness of experimental interventions that are designed to ameliorate peripheral neuropathy in laminin-alpha2-deficiency.

6. EVALUATION AND INTERPRETATION OF RESULTS

Evaluate results for statistical significance using a test that is appropriate for the experimental design and distribution of data (e.g., parametric or non-parametric t-test, Mann-Whitney, ANOVA). Any one of the many available statistics programs (e.g., InStat by GraphPad Software, San Diego CA) can be used for these analyses.

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8. ACKNOWLEDGEMENTS

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